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Application of CRISPR-Based C-to-G Base editing in rice protoplasts

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Abstract

Recently, new types of base editors, C-to-G base editors (CGBEs), that enable cytosine transversions that are unachievable with cytosine base editors (CBEs) and adenosine base editors (ABEs), have been developed in human cells. However, despite their importance in crop genome editing, the efficacy of CGBEs has not yet been extensively evaluated. In our study, based on the previously reported plant-compatible CBE and human CGBE, we demonstrated that our monocot plant-compatible CGBEs (PcCGBEs) enable cytosine transversions (C-to-G) in rice protoplasts. For all targets tested, PcCGBEs (monocot plant-compatible CGBEs) appeared to have substantial levels of C-to-G editing activity. PcCGBE showed a much higher C-to-G base editing activity and C-to-G specificity among C-to-D conversions than the mini-version of PcCGBE. Our demonstration of PcCGBE could provide a platform for the further development of enhanced CGBEs for reliable application as a new crop breeding technology.

Keywords CRISPR/Cas9, Cytosine deaminase, Uracil glycosylase, Base editing, C-to-G, CGBE, Rice protoplasts

Introduction

CRISPR/Cas9-derived base editors, cytosine base editors (CBEs) and adenine base editors (ABEs), in which base deaminases are fused to nickase Cas9 (nCas9), have been developed to convert cytosine or adenine into thymine or guanosine [1, 2]. These base editors have proven to be promising tools for crop breeding precision to confer desirable agronomic traits [3]. Li et al. developed a monocot ABE that precisely enables A-to-G conversion in rice and wheat [4]. In addition, Zong et al. developed a monocot CBE that efficiently achieves C-to-T conversion in rice, wheat and maize [5]. CBEs and ABEs often generate C-to-G substitutions via uracil glycosylation, triggering the base excision repair (BER) pathway, which serves as a basis for the recent development of C-to-G base editors (CGBEs) [6, 7]. CGBEs were developed by modifying CBEs to enhance uracil glycosylation and promote the BER pathway [8–11]. Zhao et al. developed two types of glycosylase base editors that induced C-to-A and C-to-G transversions [11]. First, they constructed a C-to-A base editor fusing nCas9 with uracil N-glycosylase (UNG) at the N-terminus and activation-induced cytidine deaminase at the C-terminus to enable C-to-A transversions in *Escherichia coli*. To construct a C-to-G base editor, they fused nCas9 with APOBEC1 (Apolipoprotein B mRNA editing enzyme catalytic subunit 1; cytidine deaminase) at the N-terminus and UNG at the C-terminus to allow highly efficient C-to-G transversions at the sixth position of the protospacer in human cells. Kurt et al. developed another CGBE with reduced undesired base substitutions and indel mutations in human cells [9]. They sequentially

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fused UNG and APOBEC1 at the N-terminus of nCas9 to achieve C-to-G transversions with a high editing efficiency. In contrast to the abovementioned CGBEs, Chen et al. developed a different type of CGBE in human cells [8]. They constructed ACX (APOBEC1-nCas9-XRCC1) fusing nCas9 with APOBEC1 at the N-terminus and XRCC1 (X-ray repair cross complementing 1), a DNA repair protein, at the C-terminus to accomplish highly efficient C-to-G transversions by facilitating base excision repair. These novel base editors broaden the range of base editing from transition to transversion substitutions, and may target more complex edits than single-base editors can achieve. Recently, several plant CGBEs have been applied to several plant species, such as rice, tomato, and poplar [12–14]. Sretenovic et al. developed plant CGBEs harboring Cas9 variants with different PAM requirements and XRCC1 [12]. Tian et al. developed rice CGBEs containing Anc689 deaminase and rice UNG [13]. In addition, Zeng et al. developed rice CGBEs fused with evoFENRY and Cas9-NG [14]. Nevertheless, the limited number of target sites for plant CGBEs was only evaluated, and C-to-G base editing activity was much lower than mammalian CGBEs as well as CBE and ABE. Therefore, the editing efficiency of plant CGBEs needs to be enhanced for versatile applications via further improvements.

Whether CGBEs can be adapted for monocotyledonous plants, such as rice, is not yet widely assessed. Here, we report the application of plant-compatible CGBEs for C-to-G transversions. Our study has demonstrated our monocot plant-compatible CGBEs enabling cytosine transversions (C-to-G) in rice protoplasts.

Results and discussion

We sought to generate a PcCGBE based on a previously reported plant base editor (PBE) [5] and human CGBE [9]. PBE consists of an N-terminal fusion of the rice codon-optimized rAPOBEC and nCas9 (D10A) with a C-terminal UGI (uracil glycosylation inhibitor) fusion under the control of maize ubiquitin 1 (Ubi-1) promoter (Fig. 1a) [5]. Based on the architecture of the reported human "miniCGBE" [9], we constructed "miniPcCGBE" by removing a UGI segment from the PBE while retaining other segments (Fig. 1a). We anticipated that miniPcCGBE might exhibit C-to-G base-editing activity, as the absence of UGI would lead to a preference for the BER pathway. Next, we constructed "PcCGBE" by adding the rice codon-optimized UNG (uracil N-glycosylase) at the N-terminal of miniPcCGBE (Fig. 1a), expecting that active uracil glycosylation might enhance the BER pathway, thus leading to preferential C-to-G base editing activity as observed in human cells [9, 11]. Four gRNAs targeting endogenous rice genes, *OsAAT*, *OsALS2*, *OsCKX2*, and *OsSPL14*, were designed and prepared in separate plasmids (Fig. 1b) [5], in which the OsU3 promoter can drive each gRNA expression in rice protoplasts.

Rice protoplasts were co-transfected with the plasmid harboring miniPcCGBE or PcCGBE, as well as the plasmid harboring each gRNAs, via the PEG-mediated method [15]. Targeted deep sequencing after 60 h of incubation post PEG treatment revealed that both miniPcCGBE and PcCGBE showed substantial levels of C-to-G editing activities at the C6 (cytosine at residue 6) positions for all tested targets (Fig. 1c). PcCGBE presented the highest C-to-G base-editing efficiency of up to 0.28% (Fig. 1c). In contrast, miniPcCGBE showed only 0.06% C-to-G base editing activity, which is far less than the activity of PcCGBE, but still an enhancement from the original PBE (0.02%) (Fig. 1c). Although specific C-to-G base editing activities were enhanced by serial modification from the original PBE, the overall enzymatic activities decreased significantly. For example, C-to-T base editing activities at the C6 position of the *OsCKX2* target site from PBE were at least fourfold higher than the C-to-G base editing activities of PcCGBE and miniPcCGBE (Fig. 1d), suggesting that the BER pathway in plants might not be as robust as that in the mammalian system.

PcCGBE showed a range of C-to-G base-editing activities depending on the target sites. The highest activities observed were up to 0.55% for targeting *OsCKX2*, whereas targeting *OsALS2* resulted in 0.12% (C6) (Fig. 1e). The C-to-G editing activities did not necessarily conform to the original PBE activities with the same corresponding gRNAs (Fig. 1e), suggesting that there might be another innate rate-limiting step in rice cells for C-to-G base editing, which likely explains the overall loss of base-editing activities observed (Fig. 1d). The measured active windows of C-to-G base editing from the PcCGBE application revealed a pattern (C4–C8) similar to those from the corresponding PBE applications (Fig. 1e). However, except for *OsCKX2* targeting, we did not observe strictly discrete C-to-G editing patterns exclusively on C6, as observed in the mammalian system, where CGBE or GBE have been reported to induce C-to-G base transversion exclusively at C6 [9, 11].

We analyzed the number of indels that had been generated from PBE, miniPcCGBE, and PcCGBE applications within the protospacer sequences (Fig. 1f). As expected, PBE rarely generated indels within the targeting windows (Fig. 1f), supporting the notion that UGI results in a bias toward mismatch repair rather than the BER pathway. Indel frequencies from PcCGBE were approximately tenfold higher than those from miniPcCGBE, consistent with their capability for uracil glycosylation, thus likely reflecting the different C-to-G outcomes. Ratios between

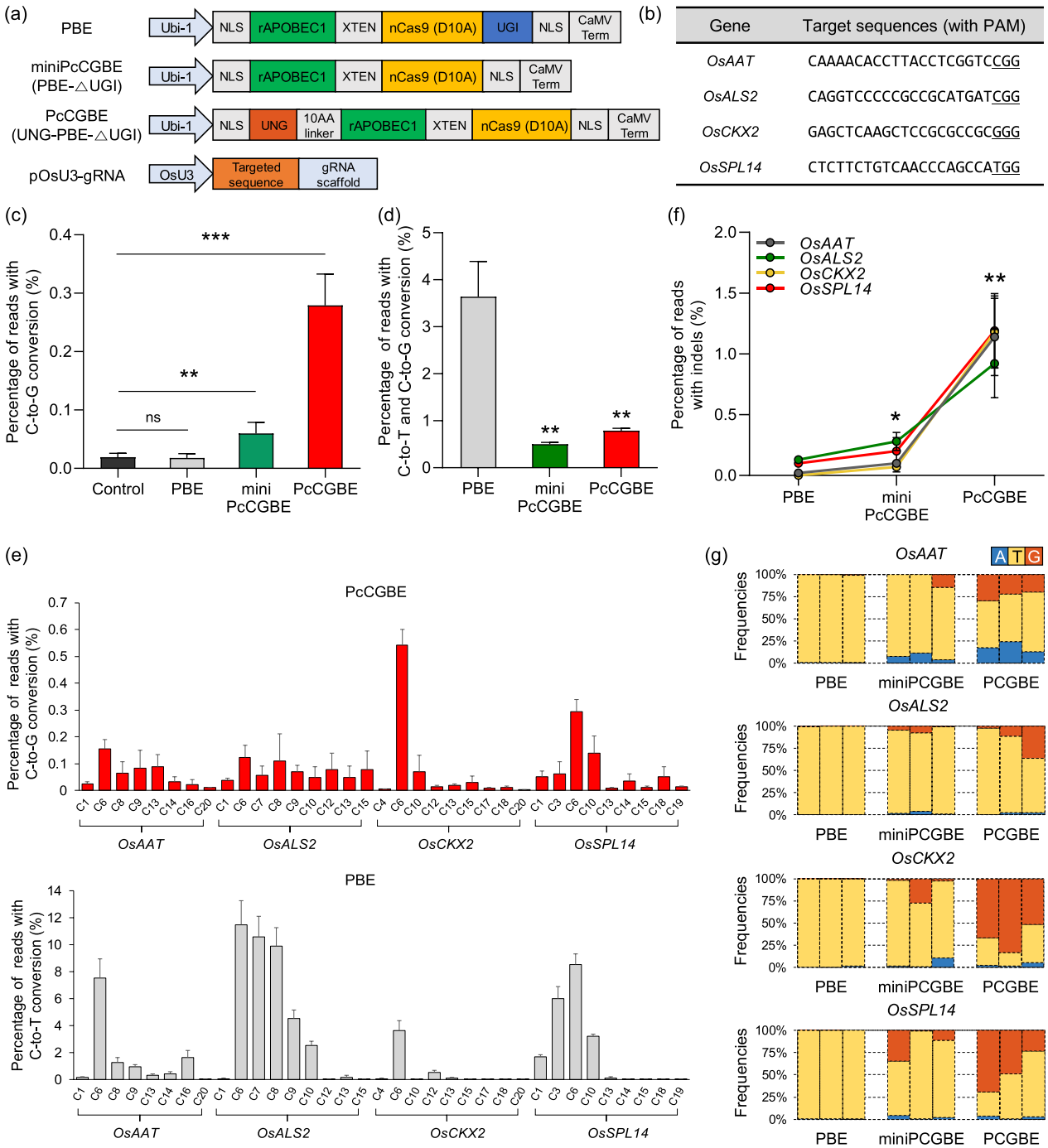

Fig. 1 Evaluation of C-to-G base editing in rice protoplasts. **a** Construction of C-to-G base editors. XTEN, a 16-aa linker; UGI, uracil glycosylase inhibitor; UNG, *E. coli* uracil glycosylase. **b** Target sequences. **c** Average C-to-G base editing efficiencies at C6 positions of four targets. Control, PEG-treated protoplasts. The *p* values were determined by two-tailed Student's t-test. ns, not significant ($p > 0.05$); $^{**}p < 0.01$, $^{***}p < 0.001$. **d** Frequencies of C-to-T and -G conversions at *OsCKX2*. **e** Frequencies of C-to-G or -T conversions using PcCGBE and PBE at all cytosines in four targets. **f** Indel frequencies in four targets. **g** Three replicas of C-to-D ratios at C6 positions of four targets. In (c), (d), (e), and (f), Values are the mean ± SEM. In (d) and (f), statistical differences were tested using one-way ANOVA analysis (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

C-to-G, C-to-A, and C-to-T conversions might represent the repair processes that were dominant during PcCGBE, miniPcCGBE, and PBE applications. PcCGBE showed increased overall C-to-G conversions at all tested targets, mostly up to 85% in *OsCKX2*, in contrast with the cases of PBE presenting exclusively C-to-T conversions, as well as marginal increase in C-to-G conversions from miniPcCGBE (Fig. 1g). These results suggest that the addition of UNG to PcCGBE might drive the BER pathway significantly once uracil glycosylation occurs.

We constructed plant-compatible C-to-G base editors by removing UGI and/or adding UNG to determine their activities at several endogenous loci in rice protoplasts. However, a range of different activities was observed depending on the target loci, and the overall base editing activities were significantly reduced compared with the original PBE. Our results raised several interesting points even with low C-to-G transversion activities. We followed the procedures of constructing the human CGBE versions reported, thereby maintaining the same architectures and functional rationales in our plant versions of CGBEs. Unfortunately, our CGBEs did not show a significant C-to-G conversion rate, contrary to our expectations. However, the C-to-G activities shown in our results seem *bona fide* because the measured activities are statistically valid, and the specific patterns of C-to-G conversions in C6 positions were also pronounced. Outcomes of CRISPR activities, in the end, vastly rely on their endogenous DNA repair pathways in tested organisms. Therefore, the results indicate that the low C-to-G editing activities might have something to do with the endogenous DNA repair pathways in plant cells. We noted that the BER pathway might not be preferred to repair the abasic sites via CGBEs in rice cells, instead promoting indel generation. In this regard, we propose that we might need to incorporate plant-specific factors either promoting more BER pathway or preventing indel generation. For instance, incorporation of reported R33A [9] or additional factors that can suppress indels might enhance C-to-G base editing activities.

Our PcCGBE will provide a platform for the further development of enhanced CGBEs that can be reliably used in new plant breeding technologies.

Materials and methods

Vector construction

The vector construction of miniPcCGBE and PcCGBE in this study was modified from pnCas9-PBE [5]. The pnCas9-PBE was used as a control in this study. The pnCas9-PBE was digested with *MluI* and *XmaI* to remove UGI to produce the miniPcCGBE. To construct PcCGBE, *E. coli* uracil N-glycosylase (UNG) was codon-optimized for *Oryza sativa* and synthesized commercially (Macrogen, Korea). The miniPcCGBE was digested with *BamHI* and *PmlI* to insert UNG into the maize ubiquitin 1 promoter. The amplified UNG and linker peptides were ligated into the digested miniPcCGBE. For guide RNA cloning, the primer pairs designed for the target site were annealed and ligated into the AarI sites downstream of the OsU3 promoter in the pUC57 vector (Additional file 1: Table S1) [5].

Protoplast isolation and transient expression

Polyethylene glycol-mediated protoplast transformation was performed as previously described [15]. Rice seedlings (*Oryza sativa* cv. Kitaake) were grown in the dark for 10 days and then transferred to light for 10 h. The protoplast density was calculated under a microscope using a hemocytometer (Marienfeld, Germany) and adjusted to 7.0×10^7 protoplasts/mL. The protoplasts (2×10^6 cells) were mixed with plasmid DNA (10 μg per construct) harboring PBE, miniPcCGBE or PcCGBE, as well as the plasmid harboring each guide RNA. Transformed protoplasts were incubated at 25 °C for 60 h. After incubation, protoplasts were collected to extract genomic DNA for a deep amplicon sequencing assay.

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted using a DNAzol kit (MRC, USA). The targeted sites were amplified using specific primers (Additional file 1: Table S1) and KOD DNA polymerase (TOBOYO, Japan), and the amplicons were purified using Expin Gel SV (Geneall, Korea).

Amplicon deep sequencing

The PCR products were sequenced using the MiniSeq system. Amplicon sequencing was done in triplicate for each target. Target sites in the sequenced reads were examined for C-to-T or C-to-G substitutions and indel frequencies. Gene analysis was performed using the CRISPR RGEN tools.

Abbreviations

CGBE	C-to-G base editor
PcCGBE	Monocot plant-compatible CGBE
UNG	Uracil N-glycosylase
APOBEC1	Apolipoprotein B mRNA editing enzyme catalytic subunit 1
BER	Base excision repair
PBE	Plant base editor

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00775-5.

Additional file 1: **Table S1.** Oligonucleotide primers used in this study.

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Author contributions
JL, NO, JYY, and CJ designed the study; HSC, JL, and NO performed the experiments; JKS, JHK, NO, JL, JYY, and CJ performed data analyses; JL, NO, JYY, and CJ wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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